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Molecular Biology of Aging, pages 365–378

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EXPRESSION OF HUMAN CHIMERIC TRANSFERRIN GENES[1]

Gwendolyn S. Adrian*, Robert Riehl*+, Damon C. Herbert*, Frank J. Weaker*, Erle K. Adrian*, LeAnn K. Robinson*, Christi A. Walter*, Carlton A. Eddy+, Carl J. Pauerstein+, Funmei Yang* and Barbara H. Bowman*

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ABSTRACT The study of expression of human chimeric genes during the aging process will be carried out by analyzing the developmental and tissue specific expression of the human transferrin (*TF*) gene fused to the bacterial *CAT* (chloramphenicol acetyltransferase) gene in the background of aging transgenic mice. The work required to identify DNA sequences that demonstrate tissue specific activity *in vitro* is summarized. Transient expression of a hybrid fusion gene composed of the 5′ region of the human *TF* gene fused to *CAT* has been analyzed in transfected human hepatoma, osteosarcoma (U-2OS) and HeLa cells. The hepatoma cell lines had previously been shown to synthesize transferrin. This study is the first to demonstrate transferrin synthesis in U-2OS cells. Gene transcription results indicate that a DNA region 1200 bp 5′ to the *TF* transcription start site contains sequences that convey tissue specificity in hepatoma and osteosarcoma cells. However, when hepatoma and U-2OS cells were transfected with a *TF-CAT* fusion gene containing a

[1]This work was supported by grants from the American Heart Association-Texas Affiliate Grant 86G-371, NIH Grants HD10202, GM33298 and AG06650, AG06872 and AG00165.

5′ flanking sequence of 3900 bp, gene expression decreased three-fold, suggesting a negative regulatory region. Because of these results the human *TF* gene constructs containing 0.67 kb, 1.2 kb and 3.9 kb of the 5′ sequence fused to the *CAT* gene have been chosen for study in transgenic mice during development, maturity and throughout the aging process.

INTRODUCTION

The goal of our laboratory is to study gene expression in the background of aging by utilizing the transgenic mouse model. Because it would be impossible to study the expression of specific genes in humans from stages of embryogenesis, to maturity and through the aging process, the transgenic mouse model was selected. It permits us to insert a defined human gene construct randomly into a mouse chromosome. A study of aging in transgenic mice expedites analysis of expression of human genes throughout the life of the mouse. The transgenic mouse model provides the necessary cell lines, growth-regulating factors, early cell lineage factors and the biochemical and physiological background of aging (1,2). Mice reach old age at 24 to 30 months. The *in vivo* response of injected genes to physiological regulators can be assessed in normal differentiated tissues that are frequently difficult to manipulate in cell culture systems. The expression can be followed through embryonal and fetal development to terminally differentiated tissues. The human genes that are inserted into mouse chromosomes are passed on to the progeny of the transgenic mice in a Mendelian manner.

Transferrin is a major plasma protein (3). The transferrin gene was chosen to study first because it (a) demonstrates tissue specific expression; (b) it displays temporal expression in development, i.e., its expression appears to be increased during development and decreased during aging; (c) it responds in a characteristic manner to hormones, metals and inflammatory signals, that is, it has multiple circuits of regulation; and (d) it demonstrates conservation of function and structure, which makes it ideal to study during mammalian development and aging. Because of its iron-binding function, expression

of this gene is required for cell proliferation and for synthesis of iron-containing proteins.

(a) Transferrin's tissue specific expression includes the liver, the choroid plexus (4) and oligodendrocytes of the brain (5), the Sertoli cells of the testis (6), helper-inducer lymphocytes (7) and the lactating mammary gland (8). Transferrin is also produced in one line of primitive osteoblast-like bone cells, the U-20S osteosarcoma cell line.

(b) In normal mice and rats, the endogenous transferrin gene expression begins during embryogenesis in the visceral yolk sac (9), later is expressed in liver; transferrin is present only in low concentrations in the brain of the newborn but increases as the animal matures.

(c) In the circulation, transferrin is decreased during iron overload (10) and increases during iron deficiency (11). During inflammation circulating transferrin concentrations increase but somewhat later in time than most acute phase reactants (12). Transferrin concentrations increase with estrogen supplementation and with pregnancy (13). Nothing is known about the modulation of transferrin in brain. Transferrin's concentration in Sertoli cells increases with hormone supplementation. This includes testosterone, insulin and FSH (14). In mice circulating transferrin decreases with age. We find in our studies that this appears to be a result of decreased level of the steady state transcription of the mouse transferrin gene (3).

(d) Transferrin is a member of a conserved family of genes that have remained linked on the same chromosome for hundreds of millions of years (15). Transferrin is the product of an ancient intragenic duplication that led to homologous carboxy and amino domains, each of which binds one ion of ferric iron. The function of transferrin, to carry iron to cells throughout the body, has also been conserved throughout vertebrate evolution. Transferrin carries iron into cells by receptor-mediated endocytosis. Iron is dissociated from transferrin in a nonlysosomal acidic compartment of the cell. Provision of intracellular iron for synthesis of ribonucleotide reductase, an enzyme that catalyzes the first step of DNA synthesis, is required for cell division. After dissociation of iron, transferrin and its receptor return undegraded to the extracellular environment and the cell membrane, respectively. Transferrin, probably because of its conserved

function as an iron transporter, is a growth factor required for the proliferation of normal and malignant cells.

While we and others have characterized extensively many aspects of plasma protein gene structure and expression, relatively little is known about the cis-regulatory DNA sequences involved in the developmental regulation of their gene expression during generation of transgenic animals. From results of sequencing and correlating DNA sequences with gene expression, it is known that conserved sequences of DNA often located in the 5′ region, in front of the coding region of the gene, serve to drive the expression of the gene at specific developmental stages and in specific tissues. DNA sequences have been identified that respond to metals, to mitotic signals and to hormonal receptors (16-19).

Nuclear proteins that can vary from tissue to tissue and presumably in each developmental stage through aging, attach to the cis-regulatory elements and affect the transcription of the gene. This is thought to be related to the relocation of chromatin structure. Our goal is to use the transgenic mouse to test different constructs of the regulatory regions of the human plasma protein genes to identify DNA sequences that respond to developmental signals, including those in the aging process.

To begin to experimentally define these regulatory sequences, we have taken the approach of introducing cloned genes with defined sequences of the regulatory region into the mouse germ line and comparing their expression in the resulting transgenic animals. In transgenic mice, the expression of the majority of introduced genes demonstrate tissue specificity, and the level of expression resembles their endogenous counterparts, in this case the mouse's own transferrin gene.

REGULATORY REGIONS OF HUMAN TRANSFERRIN GENE

The human transferrin gene has been characterized in our laboratory (20) and the cDNA was used to probe and identify the human *TF* gene from a human genomic library (21). DNA sequences identical or closely homologous to sequences identified with regulatory functions in genes other than *TF* include glucocorticoid receptor elements (16), metal regulatory elements (17), hepatic nuclear

factor-1 binding site (18) and a sequence found in γ-interferon and interleukin-2 that may be important for lymphocyte proliferation (19). The presence of the conserved sequences that match cis-regulatory elements in other genes is shown in the 5′ region of the human *TF* gene (Fig. 1).

Using a 5′ fragment of full-length human TF cDNA (20) as a probe, a lambdaphage clone was isolated that contained the 5′ end of the human *TF* gene including approximately 3.9 kb of the 5′ flanking region (21). The transcription start site of the *TF* gene has been defined by S1 nuclease analysis and primer extension (22). To investigate gene transcription controlled by *TF* regulatory regions, plasmids were constructed that contain *TF* 5′ flanking DNA fused to the *CAT* structural gene from *E. coli* (23,24). Human hepatoma cell lines, the U-20S osteosarcoma line and HeLa cells have been transfected with these plasmids to examine *CAT* expression controlled by defined 5′ flanking regions of the *TF* gene. These experiments indicate that DNA sequences within 1200 bp of the *TF* translation start site contain regions that convey tissue-specificity. Evidence was also obtained for a negative regulatory region located in the *TF* flanking chromosomal DNA between 1200 and 3900 bp 5′ to the *TF* coding region.

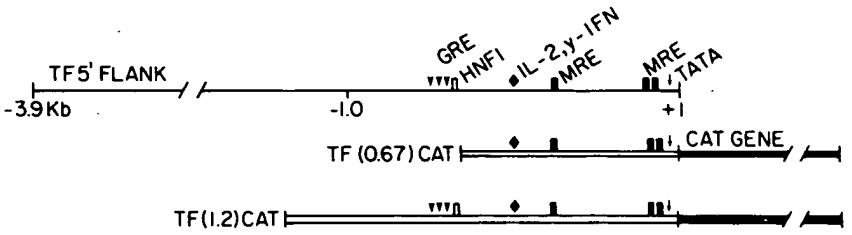


FIGURE 1. *TF-CAT* fusion genes. Diagram indicates portions of the human *TF* 5′ flanking region and potential cis-regulatory elements that are included in pTF(0.67)CAT and pTF(1.2)CAT. Regulatory elements homologous or identical to those functional in other genes include metal regulatory elements, MRE (17), glucocorticoid receptor elements, GRE (16), a sequence found in gamma-interferon and interleukin-2, γ-IFN, IL-2 (19), and hepatic nuclear factor-1, HNF-1 (18).

EXPERIMENTAL METHODS

Human hepatoma cell lines Hep3B2 and HepG2C (25) were obtained from Dr. Gretchen Darlington (Baylor College of Medicine, Houston, TX). Plasmids pSVOCAT and pSV2CAT constructed by Gorman et al. (23) were obtained from Dr. Bruce Howard (National Cancer Institute, Bethesda, MD). U-2OS and SaOS-2 cells were from American Type Culture Collection (Rockville, MD). G2 and HS27 cells were obtained from Dr. Peter Sheridan (University of Texas Health Science Center, San Antonio, TX).

Hepatoma and HeLa cells were cultured in minimal essential media: Waymouth's 87/3 (Gibco) 3:1 v/v plus 10% fetal calf serum. The cells were transfected with 10 μg/plate of plasmid DNA by the $Ca_3(PO_4)_2$-DNA coprecipitation technique (24). Transient expression was analyzed 40 hrs after transfection by CAT enzyme assay as described by Gorman et al. (23).

Immunoprecipitations were carried out following the procedure of Silverstone et al. (26) modified as previously reported (27). The lysates were labeled with ^{35}S-cysteine (1000 Ci/mmol), 0.2 mCi/T150 flask, for 45 min.

RESULTS

Construction of TF-CAT Plasmids.

Plasmids were constructed that contain *TF* 5′ flanking regions fused to *CAT* coding regions. The *TF* 5′ flanking regions were obtained from the plasmid pTFpro. This plasmid contains *TF* exon I, 3.9 kb of 5′ flanking DNA and 0.5 kb of intron I DNA cloned into the *Bam*HI and *Eco*RI sites of pUC8. Plasmid pTFpro was cut with *Sma*I and digested with Bal 31 to obtain plasmids that lacked a translation start site but maintained a transcription start site. Digested DNA was closed by ligation and recloned in *E. coli* SE10. The plasmids produced were analyzed by restriction endonuclease digestions (Fig. 2a). Two plasmids, J248-18 and J248-19, were identified that had the desired modification. Sequencing the fusion joints defined the DNA corresponding to the 5′ untranslated region of the TF mRNA and the pUC8 polylinker region that had been joined. Plasmid J248-19 was the source of the 670 bp *Hind*III fragment and 1200 bp *Hinc*II fragments that were

used in constructing pTF(0.67)CAT, pTF(0.67)CAT(REV), pTF(1.2)CAT and pSVTFCAT (Fig. 2b). Plasmid J248-18 was used in construction of pTF(3.9)CAT. In this plasmid, the 1.5 kb *Stu*I to *Bam*HI fragment of pSV2CAT which contains the *CAT* gene and SV40 polyadenylation signal was inserted into the *Bam*HI site of J248-18. *Bam*HI sites in the insert and J248-18 had been blunted by filling in the ends using reverse transcriptase. Attempts to insert a TF 5′ flanking region larger than 1.2 kb into pSVOCAT as in pTF(0.67)CAT and pTF(1.2)CAT resulted in DNA rearrangements; therefore, the alternative approach described above was used in the construction of pTF(3.9)CAT.

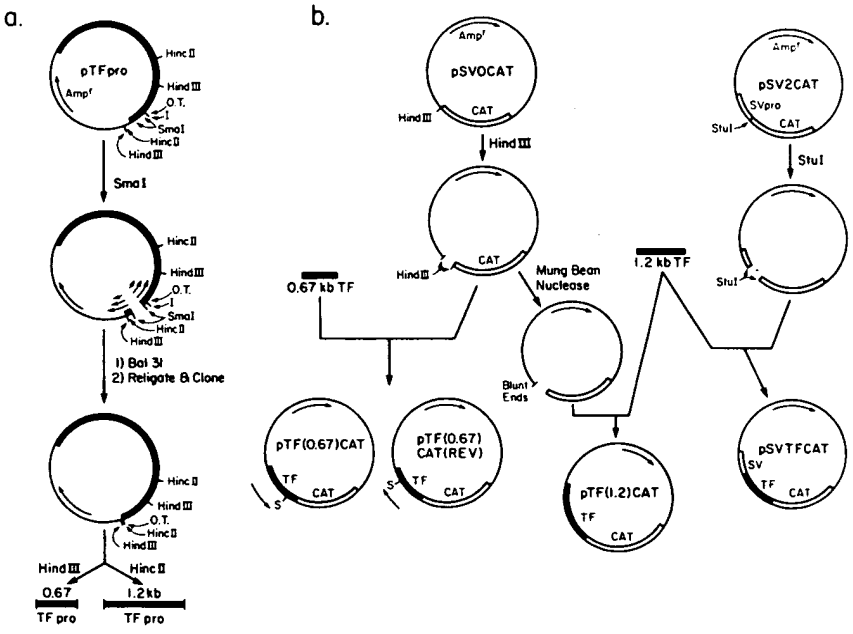


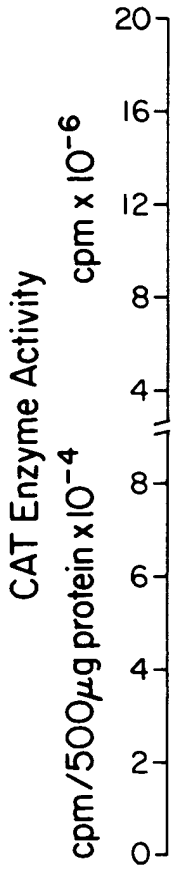
FIGURE 2. Construction of TF-CAT plasmids. TF-CAT plasmids used in transient cell transfections were constructed by first removing TF protein-coding sequences from exon 1 (A) and then inserting TF 5′ flanking sequences upstream to the CAT gene in pSVOCAT or pSV2CAT (23). See text for details.

Transfection of HeLa, U-2OS and Hepatoma Cells with pTFCAT Plasmids.

Two human hepatoma cell lines, Hep3B2 and HepG2C, are reported to synthesize transferrin (25). When these lines were transfected with pTF(0.67)CAT and pTF(1.2)CAT and assayed 40 hrs later, the CAT gene was expressed as demonstrated by CAT enzyme assay. In contrast, when the hepatoma cells were transfected with pTF(0.67)CAT(REV), a construct with the 5′ region of the *TF* gene in reversed orientation, or when they were subjected to mock transfections with no DNA, CAT enzyme activity was not detectable.

Tissue-specificity of the *TF-CAT* fusion gene expression was investigated by transfecting HeLa, Hep3B2, and U-2OS cells with pTF(1.2)CAT and pTF(3.9)CAT. The U-2OS cell line was established from a human osteosarcoma. HeLa cells do not express transferrin. With RNA blot hybridizations probed with human ^{32}P-labeled TF cDNA, TF mRNA was readily detected in 15 μg of hepatoma cell total RNA and was not detected in 46 μg of HeLa cell total RNA. The cells were also transfected with pSV2CAT as a positive control; this plasmid contains the ubiquitously-expressing SV40 enhancer and promoter fused to the *CAT* gene.

All three cell lines strongly expressed pSV2CAT indicating an effective transfection. Negligible CAT activity could be detected when HeLa cells were transfected with pTF(1.2)CAT or pTF(3.9)CAT. In contrast, the Hep3B2 and U-2OS cells consistently expressed CAT enzyme activity when transfected with pTF(1.2)CAT or pTF(3.9)CAT. Hep3B2 and U-2OS cells transfected with pTF(1.2)CAT demonstrated three- to five-fold more CAT enzyme activity than cells transfected with pTF(3.9)CAT. In this experiment transfections were carried out in triplicate and a graphic analysis of the results is shown (Fig. 3). Consistent results have been obtained in three other independent Hep3B2, HepG2C and HeLa cell transfections using different plasmid preparations of pTF(1.2)CAT. In contrast to pTF(1.2)CAT, the plasmid pTF(0.67)CAT does not show tight cell-specific control; although usually negative, in about 20% of HeLa transfections with this plasmid there is weak expression of CAT. The negative control plasmid pTF(0.67)CAT(REV) did not express CAT protein in any of the cell lines examined.



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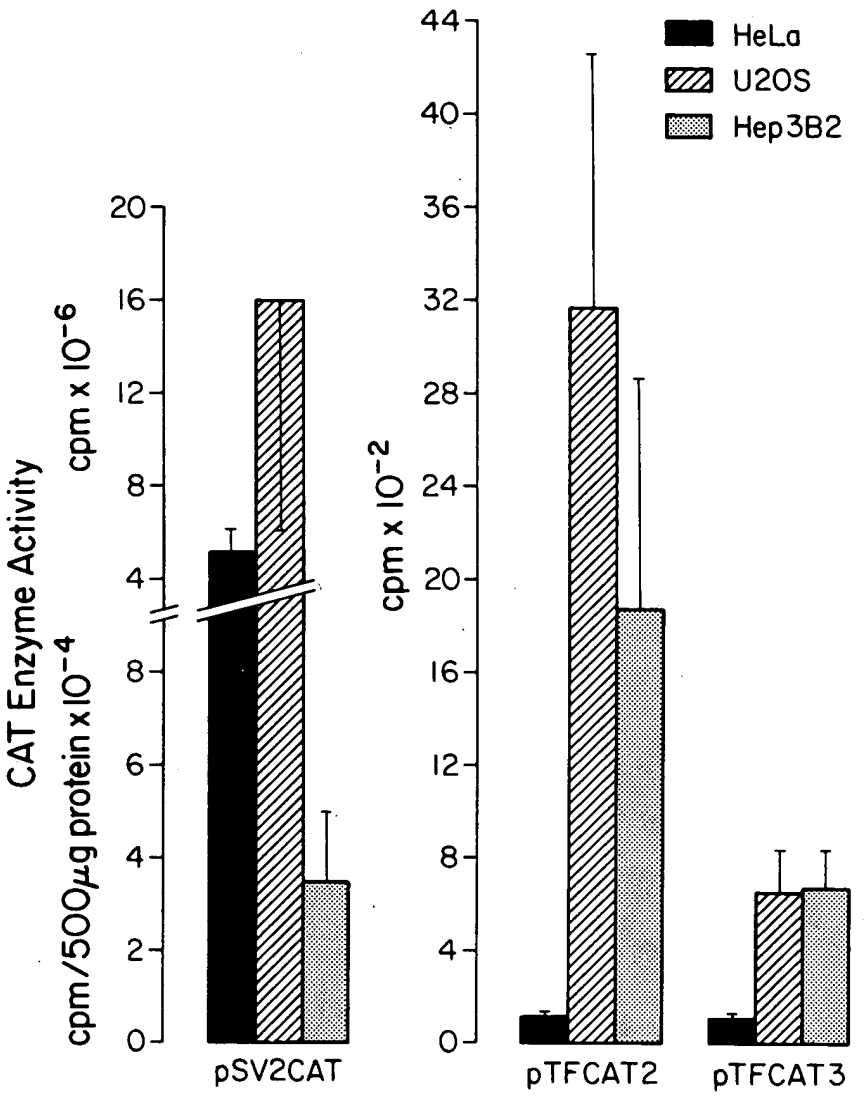

FIGURE 3. Cell-specific expression of *TF(1.2)CAT* and *TF(3.9)CAT*. HeLa, Hep3B2, and U-20S cells were each transfected, in triplicate, with pTF(1.2)CAT (pTFCAT2), pTF(3.9)CAT (pTFCAT3), and pSV2CAT, 10 μg of plasmid/transfection. Activity is expressed as cpm of acetylated [^{14}C]chloramphenicol product/500 μg protein.

Expression of Endogenous *TF* Gene and Other Liver Proteins Genes in the Osteosarcoma Cell Line U-2OS.

Expression of the TF-CAT plasmids in U-2OS cells was unexpected. To determine if these cells synthesize endogenous transferrin, homogenates of cells grown in [^{35}S]cysteine were immunoprecipitated with rabbit antiserum directed against human transferrin. Immunoprecipitations were also performed on two other human osteosarcoma cell lines, G2 and SAOS, a human fibroblast line HS27, HeLa, and Hep3B2 for comparison. U-2OS and Hep3B2 cells both synthesized [^{35}S]TF; all other lines failed to synthesize transferrin as shown (Fig. 4). The banding pattern for the U-2OS [^{35}S]TF differed from that of Hep3B2. Whereas ~95% of the U-2OS transferrin co-migrated with authentic human transferrin, ~5% migrated faster as a protein of about 69,000 daltons. The minor fraction was a derivative of transferrin since authentic transferrin competed with it for antibody (Fig. 4). Additional immunoprecipitations revealed synthesis by U-2OS cells of other proteins normally synthesized by the liver: ceruloplasmin, albumin, and vitamin D binding protein.

DISCUSSION

In the study described here we have prepared and tested human *TF-CAT* constructs and investigated gene expression of the constructs *in vitro*. When the *TF* promoter region was directed away from the *CAT* gene, as in transfections with pTF(0.67)CAT(REV), no CAT expression was detectable, providing evidence that the expression was driven by the *TF* promoter. Sequences of 670 bp, 1200 bp and 3900 bp of the *TF* 5′ flanking region in each chimeric gene directed transcription of the CAT gene as measured by CAT enzyme activity. CAT expression from pTF(3.9)CAT was three-fold lower than that of pTF(1.2)CAT. Diminished expression may be due to the presence of a negative regulatory region between -1.2 to -3.9 kb upstream from the coding region of the *TF* gene. Sequence in the 5′ region of another liver protein gene, the alpha-fetoprotein gene, has been correlated with diminished gene expression; a deletion of -1.6 to -4 kb resulted in a five-fold increase in expression of the gene (28).

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FIGURE 4. Synthesis of transferrin by U-20S, a human osteosarcoma cell line. Human cell lines were cultured in the presence of [^{35}S]cysteine and immunoprecipitated using rabbit anti-human transferrin. Large arrows indicate migration of purified human transferrin. **A.** Comparison of the forms of transferrin found in Hep3B2 and U-20S cells. **B.** Synthesis of [^{35}S]TF was demonstrated in U-20S cells, but not in two other human osteosarcoma cell lines, G2 and SAOS, a human foreskin fibroblast cell line HS27, or HeLa cells.

The *TF* gene is known to be expressed *in vivo* in a tissue-specific manner; a major site of synthesis is the liver. In accordance with *in vivo* results, hepatoma cells, but not HeLa cells, transfected with pTF(1.2)CAT and pTF(3.9)CAT consistently expressed CAT activity. These experiments indicate that the 1200 bp 5′ region of the human *TF* gene contains sequences required for tissue specific expression. Additional experiments will be required to define the precise cis-regulatory elements responsible for expression.

In this study, a human osteosarcoma cell line, U-20S, expressed both the endogenous *TF* gene and transfected TF-CAT plasmids. Whether this expression is characteristic of osteogenic cells in early development or is related to the malignant transformation of U-20S is not known. Two other human osteosarcoma cell lines examined did not express *TF*.

Identifying sequences of the human *TF* gene that can be used to study expression in transgenic mice is the first step to analyzing *TF* gene expression during the aging process. We presently have four lines of transgenic mice that carry the human *TF(0.67)CAT* chimeric gene and seven lines that carry the *TF(1.2)CAT* gene. Characterization of the tissue specific expression of the *TF* chimeric genes in transgenic mice is discussed elsewhere (22).

The *TF* gene model will be useful for defining cis-regulatory and trans-acting factors that are responsible for tissue specific and developmental expression. A better understanding of the molecular basis of aging should begin to emerge as more is learned about the order of gene expression during development.

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JPAB,EPAB,DWPI	l10 and l11	117	L12
JPAB,EPAB,DWPI	express$ or plasmid$ or vector$	248984	L11
JPAB,EPAB,DWPI	transferrin	1625	L10
USPT	435/69.1 and l4 not (l7 or l8)	30	L9
USPT	435/69.7 and l4 not l7	8	L8
USPT	l4 and l6	5	L7
USPT	((435/69.6)!.CCLS.)	538	L6
USPT	l3 and l4	127	L5
USPT	l1.ti,ab,clm.	344	L4
USPT	l1 same l2	956	L3
USPT	express$ or plasmid$ or vector$	417808	L2
USPT	transferrin	3588	L1

Set	Items	Description
S1	17778	"TRANSFERRIN"
S2	769	"TRANSFERRIN --GENETICS --GE"
S3	10525	DC="D12.776.124.50.800."
S4	7435	"RECOMBINANT FUSION PROTEINS --BIOSYNTHESIS --B"
S5	13306	"RECOMBINANT PROTEINS --BIOSYNTHESIS --BI"
S6	27	S3 AND S5
S7	17	S4 AND S3 NOT S6
S8	57961	DC="G5.331.370."
S9	94	S3 AND S8 NOT (S6 OR S7)
S10	194	S2 AND EXPRESS? NOT (S6 OR S7)
S11	13	S10 AND PLASMID?
S12	7	S2 AND PLASMID? NOT (S6 OR S7 OR S11)
S13	42	S3 AND PLASMID? NOT (S6 OR S7 OR S11 OR S2)